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Expression of Recombinant Norwalk-Like Virus Capsid Proteins Using a Bacterial System and the Development of Its Immunologic Detection

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The capsid protein of Norwalk-like virus (NLV) isolates NLV-36 (Mexico virus type, genogroup II [GII]), NLV-21 (Lordsdale virus type, GII), NLV-114 (untyped GII virus), and NLV-96-908 (KY89 virus type, GI) have been expressed in an *Escherichia coli* system. The expressed recombinant NLV capsid proteins, fused with maltose binding protein (MBP-rV) and thioredoxin (TRX-rV) in *E. coli* lysate, were analyzed using sodium dodecyl sulfate–polyacrylamide gel eletrophoresis. Rabbit IgG (R-IgG) in hyperimmune serum has been raised against MBP-rV-36 capsid protein and was purified before further study. Detection of TRX-rVs using an enzyme-linked immunosorbent assay (ELISA) showed that R-IgG had immunologic reactivity to GII as well as to the GI rV capsid proteins TRX-rV-36, TRX-rV-21, TRX-rV-114, and TRX-rV-96-908. Results of Western immunoblot (WB) analysis showed the same broad recognition of R-IgG when using the same samples. The results of the ELISA tests on serum samples obtained from patients involved in confirmed outbreaks of NLV proved that expressed NLV capsid proteins in *E. coli* can be detected by NLV-infected human serum. In addition, purified NLVs (LD virus types) derived from patients' stool could be detected using anti-NLV R-IgG, whereas normal R-IgG did not react when using WB. Our results strongly suggest that the immunologic detection of NLV antigens using anti-rV R-IgG is possible and seems a significant step toward simplification of an NLV detection test. ***J. Med. Virol. 60:475–481, 2000.***

KEY WORDS: human calicivirus; small, round, structured virus (SRSV); reverse transcription–polymerase chain reaction; *Escherichia coli* expression system

INTRODUCTION

Norwalk-like virus (NLV), also referred to as small, round, structured virus (SRSV) or human calicivirus (HuCV), is a major pathogenetic agent associated with food-borne outbreaks of acute nonbacterial gastroenteritis in Japan. Since the first description of Norwalk virus (NV) as an etiologic agent by Kapikian et al. [1972], viruses that are indistinguishable in morphologic features increasingly have been reported as the causative agent of clinically similar gastroenteritis outbreaks. The study of these viruses has developed slowly for several reasons: the inability to cultivate these viruses in cell culture, lack of suitable animal models for experimental purposes, a dearth of antigen (Ag) to produce widely available serologic assays, and dependence on electron microscopy for the detection of virus particles.

Owing to the results of cloning and characterization of the genome of NV [Jiang et al., 1990] and Southampton virus [Lambden et al., 1993], NLV has been classified as a member of the *Caliciviridae.* Development of reverse transcription–polymerase chain reaction (RT-PCR) by Jiang et al. [1992a] has increased our knowledge of the true extent of NLV infections worldwide. As sequence data of NLVs and classic HuCVs have accumulated, HuCVs have been divided into at least three genogroups based on sequence differences of the RNA-

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TABLE I. Norwalk-like Virus (NLV) Strains, *Escherichia coli,* and Plasmid Strains That Were Used in This Study*

Strains of NLV, *E. coli,* and vectors	Relevant characteristics
NLV-36	Similar to MX
NLV-21, -38, -2	Similar to LD
NLV-114	Between GI and GII (untyped virus)
NLV-96-908	Similar to KY89
E. coli	XL1-blue
	GI724
Plasmids	pT7 blue (R)-T TA cloning vector
	pTrx-Fus* expression vector with λPL promoter
	pMAL-c2 expression vector with tac promoter

*NLV-96-908 was detected in Ehime prefecture by Dr. Oseto, and other strains were detected in our institutes. GenBank accession numbers for strains are NLV-36, AB028244; NLV-21, AB028245; NLV-114, AB028246, and NLV-96-908, AB028247. Strains available from GenBank are Mexico virus (MX), U22498; Lordsdale virus (LD), X86557; and KY89, L23828.

dependent RNA polymerase and capsid regions of the genomes [Wang et al., 1994; Ando et al., 1995; Jiang et al., 1996]. These three genogroups include the Norwalk-like, Snow Mountain–like, and Sapporo-like virus genogroups and are also referred to as genogroups I and II and classic calicivirus, respectively [Vinje et al., 1997; Honma et al., 1998; Hale et al., 1999]. Genogroups I (GI) and II (GII) are subdivided by Noel et al. [1997] into at least 10 clusters based on their partial capsid nucleotide (nt) sequence and amino acid (aa) identities.

The subsequent expression of recombinant NV (rNV) capsid proteins in baculoviruses (and its virus-like particles [VLPs]) provided the potential for establishing an immunologic assay for the diagnosis of infections and sero-epidemiological studies [Jiang et al., 1992b]. Expression of NLV capsid proteins in baculoviruses results in spontaneous assembly of the protein into empty recombinant NLV VLPs (rVLPs). These rVLPs are similar in morphologic features and Ags to native NLV particles, but the immunogenicity of these particles is extremely high [Jiang et al., 1992b]. Several reports deal with expression of rV capsid proteins using mostly baculovirus systems, among them, recombinant Mexico virus [Jiang et al., 1995c], recombinant Lordsdale virus [Dingle et al., 1995], recombinant Toronto virus [Leite et al., 1996], recombinant Hawaii virus [Green et al., 1997], and recombinant Grimsby virus [Hale et al., 1999]. Immunoreactivity of these anti-NLV antibodies (Abs) raised against rVLPs, expressed in baculovirus systems, was limited within a small range of NLV types [Jiang et al., 1995a, 1995b, 1996]. This article describes our examination of the immunologic reactivity of rabbit IgG (R-IgG) and confirmed patients' serum samples involved in outbreaks of NLV gastroenteritis against rV capsid proteins expressed in an *Escherichia coli* system.

MATERIALS AND METHODS

Viruses, Vectors, and *Escherichia Coli* Strains

The newly isolated strains NLV-36 and NLV-114 were derived from patients' stool in sporadic food-borne gastroenteritis outbreaks and were submitted to the Division of Food Microbiology, Osaka Prefectural Institute of Public Health. Strains NLV-21, NLV-38, and NLV-2 were isolated and submitted to the National Institute of Infectious Disease (Tokyo). Strain NLV-96-908 (GI type) was kindly provided by Dr. M. Oseto of Ehime Prefectural Institute of Public Health and Environmental Science.

The pT7 blue was used as a TA cloning vector (Takara Biomedicals, Japan). In order to use the *Bam*HI site as the expression frame, pTrxFus (Invitrogen, La Jolla, CA) was slightly modified and named pTrxFus*. The expression vectors pMAL-c2 (New England BioLabs, Beverly, MA) and pTrxFus* have been used to construct fusion proteins between maltose binding protein (approximately 40 kd) and NLV capsid proteins and between thioredoxin (approximately 12 kd) and NLV capsid proteins, respectively. *E. coli* strain XL1-blue was used as host cells for both pT7 blue and pMALc2, while *E. coli* GI724 was used for pTrxFus*. These NLV and *E. coli* strains are listed in Table I together with their vectors.

Reverse Transcription–Polymerase Chain Reaction

Virus RNA was extracted from 10% phosphate-buffered saline–homogenized fecal samples using ISOGEN-LS (Nippon Gene, Japan). Procedures for extraction were executed according to the manufacturer's protocol. RT and PCR were performed using the RNA LA PCR kit (AMV), version 1.1 (Takara Biomedicals, Japan). RT was carried out at 42°C for 30 min with oligo dT-adapter primer (content of the kit) and denatured at 99°C for 5 min with a DNA thermal cycler (Perkin-Elmer ABI). PCR comprised 40 cycles with denaturation (94°C for 1 min), primer annealing (55°C for 1 min and 20 sec), and extension (72°C for 2 min) using the Gene Amp PCR System 2400 (Perkin-Elmer ABI). Primers used were 82SM (5′-CCACTATGATGCA-GATTA-3′), a slightly modification of primer 82 according to Wang et al. [1994], and M13 primer M4 (content of the kit).

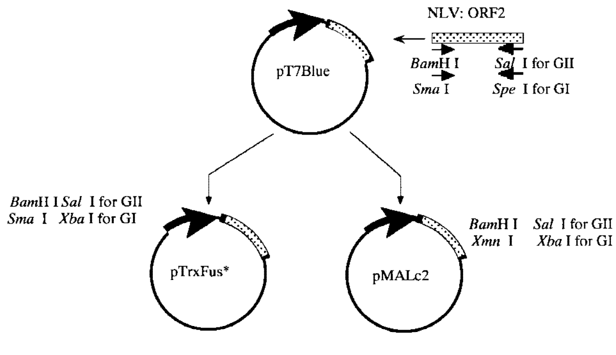

Fig. 1. Schematic diagram of the NLV ORF2 expression system.

To amplify the ORF2 region using nested PCR, the following primer pairs were synthesized in our laboratory: SRSVI-L1 (5′-ATACCCGGGATGATGATGGC-GTCTAAGGAC-3′) and SRSV I-L2 (5′-TATACTAGTT-TATWWWCGGCGCAVWCCAAGCC-3′) for GI ORF2 and SRSV II-L1 (5′-CGGGATCCATGAAGATGGC-GTCGARTSRCGC-3′) and SRSVII-L2 (5′-AGTCGACTTAYWRHRYHCKYCTRCGCCC-3′) for GII ORF2, respectively. Nested PCR comprised 35 cycles, the first five cycles of denaturation (94°C for 1 min), primer annealing (37°C for 1 min 20 sec), and extension (72°C for 2 min). The subsequent 30 cycles were similar to the first five cycles, except for primer annealing at 58°C (GI) and primer annealing at 52°C (GII).

Cloning and Expression

Figure 1 shows the schematic diagram of the NLV ORF2 cloning and expression system. ORF2 was amplified by using primer pairs that contain restriction enzyme (RE) sites (also shown in Fig. 1). The PCR products were inserted into pT7 blue. Both strands of ORF2 were sequenced with M13 primers M1 and RV (Takara Biomedicals, Japan) and designed primers for each strain by using the PRISM Dye-Deoxy Terminator Cycle Sequencing kit (Perkin-Elmer ABI), as described previously by Cauchi et al. [1996]. At least three clones of each strain were sequenced. The GenBank access numbers are indicated in the legend of Table I. Strains NLV-36, NLV-21, NLV-114, and NLV-96-908 were selected for expression experiments after analysis of the sequence data. ORF2 fragments were obtained by RE digestion with either *Sma*I and *Spe*I (GI) or *Bam*HI and *Sal*I (GII) from amplified clones of pT7 blues and were subsequently purified using agarose electrophoresis. Convenient RE sites on both sides of ORF2 were used for cloning (Fig. 1). The pMAL-c2 was digested with either *Bam*HI and *Sal*I or *Xmn*I and *Xba*I, while the pTrxFus* was digested with either *Bam*HI and *Sal*I or *Sma*I and *Xba*I, for ligation with the purified GI or GII ORF2 fragments. The ligation mixture was then used to transform XL1-blue or GI724 competent cells.

Induction and Purification of *Escherichia Coli*–derived MBP-rV and TRX-rV Capsid Protein Fragments

The resulting colonies carrying the expression constructs were incubated and induced with IPTG for XL1-blue and tryptophan for GI724, according to the manufacturer's protocol. Expression of the fusion proteins was analyzed using Coomassie brilliant blue (CBB)–stained sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) gels. Purification of maltose binding protein (MBP) fusion proteins was performed according to the manufacturer's protocol. Briefly, purification of the MBP-rV capsid proteins was carried out using an amylose resin column with the centrifuged lysate of the sonicated sample in lysis buffer. Purification of thioredoxin (TRX) fusion proteins was done using sonication and centrifugation twice, in 200 mmol/L Tris buffer (pH 7) containing 1 mmol/L EDTA, 1 mmol/L 2-mercaptoethanol (ME), and protease inhibitors. The centrifuged lysate containing TRX-rV capsid proteins was obtained by further sonication of dissolved sediments in 200 mmol/L Tris buffer (pH 12) containing 1 mmol/L EDTA, 1 mmol/L 2 ME, and protease inhibitors. These partially purified fusion proteins were then used for further experiments.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Immunoblotting Assays

Purity and concentration of the fusion proteins were estimated from CBB-stained SDS-PAGE gels. Proteins were diluted in sample buffer and boiled for 5 min. The purification of virus in stool was performed according to a method described previously [Utagawa et al., 1994]. The re-soluble proteins were transferred to a Nitran membrane (Schleicher and Schuell, Germany) using Western transfer buffer (trizmabase, 3.0 g; glycine, 17.1 g; methanol, 247.8 ml/L) with semidry equipment BP-312 (Bio-Craft, Japan). The membranes were saturated overnight by incubation at 4°C with block ace solution (BA) (Snow Brand, Japan). After blocking, the membranes were incubated for 1 hr at 37°C with either anti-MBP-rV-36 R-IgG or normal R-IgG diluted at 1:5,000 in BA buffer (a 1:5 BA dilution in Tris-buffered saline with 0.05% Tween 20). After three washes with 0.05% Tween 20 in Tris-buffered saline (TTBS), bound Abs were detected using horseradish peroxidase (HRP)-conjugated anti-R-Igs (Dako Japan, Japan) diluted at 1:1,000 in TTBS. TMB [3,3′,5,5′-tetramethylbenzidine] membrane peroxidase substrate (Kirkegaard and Perry, Gaithersburg, MD) was used as the substrate for color development.

Enzyme-linked Immunosorbent Assay

Immunoreactivity of R-IgG and patients' sera from NLV outbreaks against either TRX-rVs or MBP-rVs was determined by direct enzyme-linked immunosorbent assay (ELISA). Microplates E.I.A./R.I.A. no. 3591 (Costar, Cambridge, MA) were coated with 1 μg of the

different Ag TRX-rV and TRX or MBP-rV and MBP fragments and incubated overnight at 4°C in carbonate buffer (15 mmol/L of sodium carbonate, 35 mmol/L of sodium bicarbonate, and 0.05% of sodium azide at pH 9.6). The microplates then were washed four times with TTBS. Microplates were incubated with either biotinated anti-NLV-36 R-IgG (B-R-IgG) at various concentrations adsorbed with 10 μg/ml TRX for 30 min at room temperature or with various dilutions of patient sera (1:250 to 1:16,000) for 1 hr at 37°C in BA buffer. Vectastain ABC (Vector Laboratories, Burlingame, CA) or HRP-labeled anti-human IgG, A, and M (Dako Japan), diluted at 1:1,000 in TTBS, were used as conjugates. After incubation with Vectastain ABC for 30 min or with HRP-labeled anti-human IgG, A, and M for 1 hr at 37°C, bound Abs were detected by adding TMB as substrate and incubating for 30 min at room temperature. The enzyme reaction was stopped with 1 mol/L phosphoric acid, and the absorbance was measured at 450 nm in an ELISA microplate reader, model 550 (Bio-Rad).

RESULTS

Expression and Purification of *Escherichia Coli*–derived MBP-rV and TRX-rV Capsid Protein Fragments

Production of MBP-rV and TRX-rV capsid protein fragments in *E. coli* was analyzed by CBB staining of SDS-PAGE gels (Fig. 2). The yield of the purification recombinant protein was estimated as 5.2 mg/200 ml for MBP-rVs and 11.2 mg/200 ml for TRX-rVs, by visual comparison with known quantities of bovine serum albumin in CBB staining of SDS-PAGE gels. The partially purified recombinant MBP-rV, and TRX-rV fragments have proteolytic fragments (as can be judged from Fig. 2).

Immunoreactivity of the Anti-MBP-rV-36 Rabbit IgG Against Various Recombinant Viruses by Western Blot and Enzyme-linked Immunosorbent Assay

To examine the immunoreactivity of anti-MBP-rV-36 R-IgG against different types of rVs, WB and ELISA were carried out (Fig. 3). TRX fusion rVs were used for both methods. Both rV GI (96-908) and rV GII (36, 21, and 114) were detected by anti-MBP-rV-36 R-IgG in both tests, as shown in Fig. 3A and 3B, but pre-immune R-IgG could not detect any rVs (Fig. 3C). The reactivity efficiency toward individual rVs has been found to differ, as indicated in Fig. 3B. The conserved N terminal of NLV-36 consists of approximately 275 aa residues and was compared with NLV-21, NLV-114, and NLV-96-908. The homology was found to be 75%, 78%, and 57%, respectively, while whole capsid aa homology was 66%, 68%, and 47%, respectively. The reactivity efficiency differed from predicted aa residues, based on the 5′-end nt sequence data as well as whole nt sequence data. This probably suggests the existence of particular conformational epitope(s) or limited sequential epitope(s) in capsid residues.

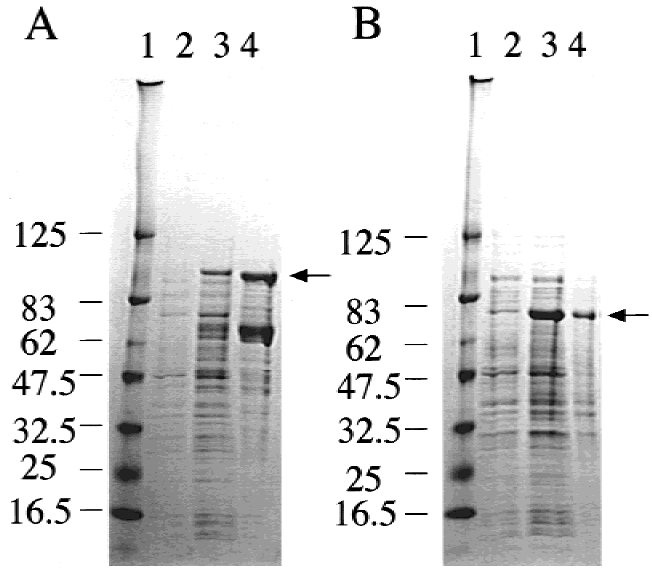

Fig. 2. The expression of the recombinant capsid proteins in *E. coli* was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis. **A: Lane 1**: marker (kd); **lane 2**: maltose binding protein (MBP) Norwalk-like virus (NLV) 36 (MBP-NLV-36) expressing *E. coli* lysate (before induction); **lane 3**: MBP-NLV-36 expressing *E. coli* lysate (after induction); **lane 4**: MBP-NLV-36 purified products. **B:** Lane 1: marker (kd); lane 2: thioredoxin (TRX)-NLV-36 expressing *E. coli* lysate (before induction); lane 3: TRX-NLV-36 expressing *E. coli* lysate (after induction); lane 4: TRX-NLV-36 purified products. The positions of the recombinant NLV capsid proteins are indicated by arrows.

Norwalk-like Virus–specific Immunoglobulins in Patients

To study whether the serum samples of NLV-infected patients detect different types of rVs, a direct ELISA was carried out; two representative changes of the pattern in the acute phase and convalescent phase are shown in Fig. 4. ELISA results showed that the serum samples of two patients detected all of the rNLVs. In addition, results indicated that both patients showed significant changes in their reactivity between the acute and convalescent phase. The isolated virus from patient 2 showed an nt sequence similar to that of the MX virus, and ELISA results suggested this patient's infection history. The same method of analysis showed that patient 1 had been infected by NLVs similar to the LD virus. Using these rVs, ELISA testing might indicate which type(s) of infected NLV(s) are found in outbreaks.

Anti-rV Rabbit IgG and Detection of Purified Norwalk-like Viruses Derived from Patients' Stool Using Western Blot

Using purified LD virus-type NLVs (21, 38, and 2), WB analysis was performed to determine whether anti-rV R-IgG detects fecal NLVs in patients, as shown in Fig. 5. The anti-rV R-IgG against rV-36 (MBP-rV-36, MX virus type) capsid proteins specifically detected purified fecal NLVs with an approximate molecular

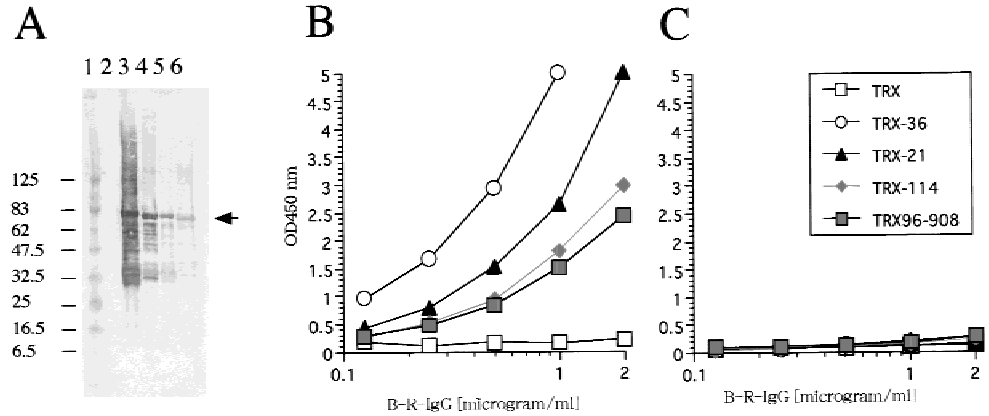

Fig. 3. The reactivity of anti-Norwalk-like virus (NLV) rabbit IgG (R-IgG) against various NLV recombinants was examined by Western blotting (**A**) and enzyme-linked immunosorbent assay (ELISA) (**B**). The data of pre-immune serum analyzed by ELISA are shown in **C**. A: **Lane 1**: marker (kd); **lane 2**: thioredoxin (TRX); **lane 3**: TRX-NLV-36 (GII); **lane 4**: TRX-NLV-21 (GII); **lane 5**: TRX-NLV-114 (GII); **lane 6**: TRX-NLV-96-908 (GI). Each recombinant was loaded 500 ng per lane. The positions of the recombinant NLV capsid proteins (TRX-NLVs) are indicated by an arrow. B: NLV recognition efficiency of the biotinated R-IgG (B-R-IgG) was examined using different types of NLV recombinants and different concentrations of B-R-IgG. C: NLV recognition efficiency of the biotinated pre-immune R-IgG.

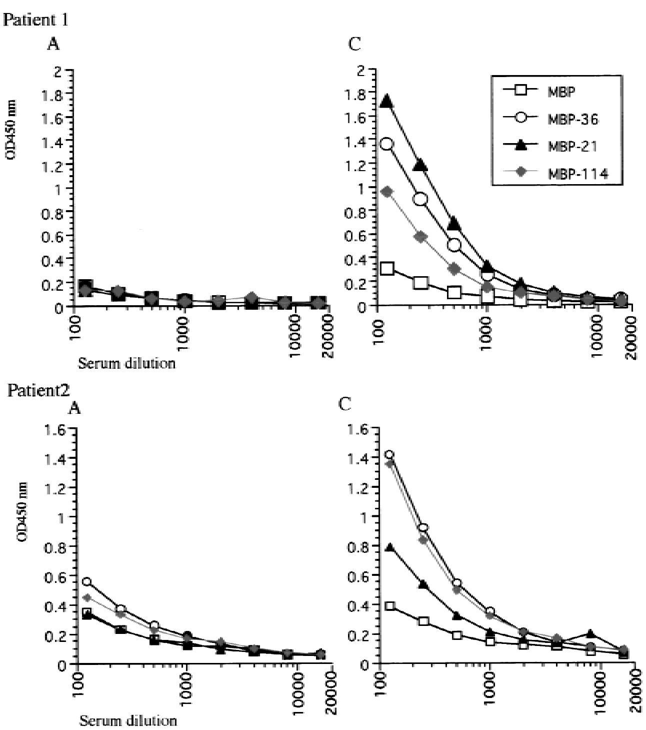

Fig. 4. The reactivity of NLV-specific immunoglobulins in the serum samples (**A:** acute phase; **C:** convalescent phase) of two patients was measured by ELISA using recombinants (maltose binding protein [MBP] as a negative control and MBP-NLV capsid proteins). Patients 1 and 3 were from different outbreaks.

weight of 60 kd, whereas normal R-IgG did not detect these NLVs.

DISCUSSION

In this study, we have constructed MBP and TRX fusion NLV capsid regions in expression vectors and expressed these as fusion proteins in *E. coli.* Anti-NLV R-IgG raised against MBP-rV-36 (MX virus type) was obtained, detecting GI and GII rV using an ELISA as well as WB. In addition, R-IgG was found to be able to detect purified fecal NLVs (LD virus type) derived from patients. These results indicate a potential application in NLV Ag detection. The ELISA results of the immunoreactivities of the patients' sera against MBP-rVs indicated that the type(s) of NLV(s) infecting an individual patient could be identified, as shown in Fig. 4. The system proposed in this article is applicable to NLV diagnosis—both specification of the type(s) of NLV(s), and epidemiological immunodetection. It was found that the TRX-rVs were not appropriate for detecting specific Igs of patients, since these sera were highly reactive to TRX.

Application of an *E. coli* expression system has the following characteristic advantages: the recombinant product can be obtained within a relatively short time (within 1 week after cloning), the expression and purification procedure is simple and easy, and expensive facilities are not required. Recombinant viral proteins, such as feline calicivirus capsid proteins, can be obtained using an *E. coli* system [Guiver et al., 1992]. However, rNLV expressed in an *E. coli* system has not been reported since Jiang et al. [1992b] established the promising rNLV VLPs in baculovirus systems, suggesting the potential for instituting an immunologic assay for both sero-epidemiological studies and diagnosis of infections. These anti-NLV Abs raised against rNLV VLPs using the baculovirus system show little cross-reactivity [Hale et al., 1999], whereas the anti-NLV Abs we obtained showed rather strong cross-reactivity. The concentration of B-R-IgG to obtain 1 OD value at 450 nm was 0.125 μg/ml for TRX-rV-36 (MX type) and 0.3 μg/ml for TRX-rV-21 (LD type). For both TRX-rV-114 (untyped virus) and -96-908 (KY89 type), this value was 0.6 μg/ml. Thus, the maximum difference among TRX-rV strains is a factor 4.8, but Abs raised against the rNLV VLP system showed a factor difference of 8 to 8,192. The minimum difference of detection

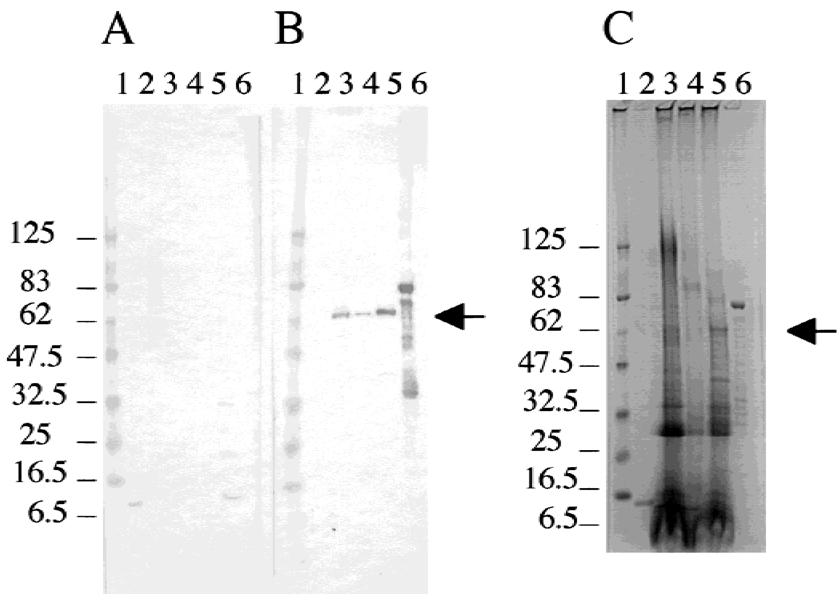

Fig. 5. Immunoreactivity of purified Norwalk-like viruses (NLVs) from patients' stool with normal rabbit IgG (R-IgG) (**A**) and anti-NLV R-IgG (**B**) by Western blotting. The resolved proteins (the same proteins used in A and B) in sodium dodecyl sulfate–polyacyrlamide gel electrophoresis gel are shown in **C**. A., B, and C: **Lane 1**: marker (kd); **lane 2**: recombinant thioredoxin (TRX); **lane 3**: purified NLV-21; **lane 4**: purified NLV-38 (Lordsdale virus [LD] type); **lane 5**: purified NLV-2 (LD type); **lane 6**: TRX-NLV-21. The positions of the purified NLV capsid proteins are indicated by arrows.

efficiency was found between NV and Grimsby virus (GRV), similar to the LD virus in the data of Hale et al. [1999]. Comparison with our data showed a factor of 2.4 for R-IgG between the MX virus and LD virus.

The reason why anti-rNLV Ab rVLPs show limited rNLV recognition using the baculovirus system is not clear. Surface representation of the three-dimensional structure of baculovirus-expressed NV capsid has been reported by Prasad et al. [1996]. It was suggested that the most variable region (the midregion) among NLVs forms the top domain, also referred to as the distal globular domain and named P1. The C-terminal portion forms the middle domain, or the central stem domain, named P2 in the rNV structure. The 250 residues of the N terminal might constitute the shell domain, named S. This indicates that the N terminal's 250 residues are not exposed to the capsid surface, since they are buried. In the case of NLV infection, it is estimated that as much as 50% of the excreted Ag is soluble protein [Greenberg et al., 1981; Graham et al., 1994]. These soluble proteins, approximately 30 kd in stool extracts and 34 kd in rNLV products, have been observed during VLP production in a baculovirus system [Jiang et al., 1992b]. They were found to be identical and unfolding the C terminal of capsid protein cleaved by trypsin using rNV. Only soluble 58-kd capsid protein is susceptible to cleavage [Hardy et al., 1995]. They also suggested the possibility that the majority of soluble proteins (C terminal) induce Abs and that these Abs may be produced predominantly following infection with NLV. Considering that rVLPs are not susceptible to cleavage, most of the induced Abs against rVLPs, using a baculovirus system, might also be C-terminal half specific.

In addition, antigenic mapping of rNLV capsid proteins, using 10 monoclonal Abs against rNLV VLPs, showed that the C-terminal of half of the capsid proteins may contain the immunodominant epitopes [Hardy et al., 1996]. Therefore, anti-NLV Abs against native NLV particles, as well as rNLV VLPs using the baculovirus system, tended to detect C-terminal regions. On the other hand, anti-NLV Abs against rNLV from an *E. coli* system may detect various regions, including both C- and N-terminal regions. Considering the most conserved regions (according to Prasad et al., [1996] between aa residues 30 and 250 in the NLV capsid sequence), it is assumed that anti-NLV Abs raised against rNLV in an *E. coli* system have a broad recognition of NLVs. In conclusion, we showed the possibility of establishing a detection system of NLV Ags, using ELISA, containing R-IgG with broad recognition of rNLVs and the possibility of confirming the diagnosis of NLV-infected patients using rVs (distinguishing the type of NLV infection in patients using rVs). The ELISA system for detecting NLV Ags from stool samples must be developed further and rVs of new isolated strains, different from established rVs, must be used to achieve these objectives.

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